Bone 56 (2013) 31–41

Contents lists available at SciVerse ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Prostaglandin E_2 acts *via* bone marrow macrophages to block PTH-stimulated osteoblast differentiation *in vitro*

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ARTICLE INFO

Article history:
Received 1 February 2013
Revised 19 April 2013
Accepted 20 April 2013
Available online 29 April 2013

Edited by: J. Aubin

Keywords:
Cyclooxygenase-2
EP4 receptor
EP2 receptor
Osteoclasts
Bone marrow stromal cells
Osteoprotegerin

ABSTRACT

Intermittent PTH is the major anabolic therapy for osteoporosis while continuous PTH causes bone loss. PTH acts on the osteoblast (OB) lineage to regulate bone resorption and formation. PTH also induces cyclooxygenase-2 (COX-2), producing prostaglandin E_2 (PGE_2) that can act on both OBs and osteoclasts (OCs). Because intermittent PTH is more anabolic in *Cox-2* knockout (KO) than wild type (WT) mice, we hypothesized COX-2 might contribute to the effects of continuous PTH by suppressing PTH-stimulated differentiation of mesenchymal stem cells into OBs. We compared effects of continuous PTH on bone marrow stromal cells (BMSCs) and primary OBs (POBs) from *Cox-2* KO mice, mice with deletion of PGE_2 receptors (*Ptger4* and *Ptger2* KO mice), and WT controls. PTH increased OB differentiation in BMSCs only in the absence of COX-2 expression or activity. In the absence of COX-2, PTH stimulated differentiation if added during the first week of culture. In *Cox-2* KO BMSCs, PTH-stimulated differentiation was prevented by adding PGE_2 to cultures. Co-culture of POBs with M-CSF-expanded bone marrow macrophages (BMMs) showed that the inhibition of PTH-stimulated OB differentiation required not only COX-2 or PGE_2 but also BMMs. Sufficient PGE_2 to mediate the inhibitory effect was made by either WT POBs or WT BMMs. The inhibitory effect mediated by COX-2/PGE_2 was transferred by conditioned media from RANKL-treated BMMs and could be blocked by osteoprotegerin, which interferes with RANKL binding to its receptor on OC lineage cells. Deletion of *Ptger4*, but not *Ptger2*, in BMMs prevented the inhibition of PTH-stimulated OB differentiation. As expected, PGE_2 also stimulated OB differentiation, but when given in combination with PTH, the stimulatory effects of both were abrogated. These data suggest that PGE_2, acting *via* EP4R on BMMs committed to the OC lineage, stimulated secretion of a factor or factors that acted to suppress PTH-stimulated OB differentiation. This suppression of OB differentiation could contribute to the bone loss seen with continuous PTH *in vivo*.

Introduction

Parathyroid hormone (PTH) is the major regulator of calcium homeostasis through its actions on bone and kidney. PTH is critical for bone remodeling, exerting both anabolic and catabolic effects on bone *in vivo* by activating the PTH1 receptor, a G-protein coupled receptor, on osteoblast (OB) lineage cells [1,2]. Intermittent PTH was the first anabolic agent approved for osteoporosis therapy in the USA [1,3]. For reasons still not completely understood, daily injections of PTH increase bone formation more than resorption, thereby increasing bone mass, while continuous infusion increases bone resorption more than formation, resulting in bone loss [4–6]. Despite the anabolic effects of PTH *in vivo* and the demonstration that PTH can stimulate OB precursors or mesenchymal stem cells (MSCs) to differentiate into OBs [2,7], it has been difficult to demonstrate osteogenic effects of PTH *in vitro*. A number of *in vitro* studies have reported that PTH present continuously in culture inhibits OB differentiation [8–11]. These observations suggest that the bone loss associated with continuous PTH is not simply the result of increased resorption but may also involve suppressed differentiation of bone-forming cells.

PTH is also a potent inducer of cyclooxygenase-2 (COX-2) and prostaglandin (PG) production, especially PGE_2, in OB lineage cells [12,13]. PGs are locally produced lipids that have receptors on both OB and osteoclast (OC) lineage cells [14,15]. PGE_2 is abundantly expressed in bone and can have important roles in skeletal metabolism. Although originally identified as a resorption agonist, PGE_2 also increases bone formation *in vivo* [16] and OB differentiation *in vitro* [14,15]. Multiple regulators of bone metabolism induce COX-2, the major enzyme responsible for PG production. For some of these, their induction of COX-2 enhances or mediates their stimulation of OB differentiation *in vitro* [14,17–19]. In addition, endogenous PGs are also necessary for normal bone repair [20] and a critical role for COX-2 and PGE_2 in triggering Wnt/β-catenin signaling in the anabolic response to

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8756-3282
http://dx.doi.org/10.1016/j.bone.2013.04.017

mechanical loading has been proposed [21]. Four G-protein coupled receptors, EP1, EP2, EP3 and EP4, are associated with effects of PGE_2. EP2 and EP4, which activate $G\alpha_s$ and stimulate cAMP formation, have predominant roles in both PGE_2-stimulated bone resorption and formation [15]. EP3 is coupled to $G\alpha_i$ and inhibits cAMP, while EP1 acts largely by increasing calcium flux and perhaps protein kinase C (PKC) [22].

Because PTH induces PGE_2 production and because PTH and PGE_2 both have major actions *via* similar $G\alpha_s$/cAMP-activated pathways [23,24], our initial hypothesis was that PGE_2 was the local mediator of some of the anabolic actions of PTH. However, we found intermittent PTH *in vivo* to be more anabolic in *Cox-2* KO mice than in WT mice, suggesting an inhibitory interaction of PTH and PGs [25]. In the current study, we extend our initial findings on the inhibitory interaction of PTH and PGs *in vitro* [26] to show that the stimulatory effect of PTH on OB differentiation in BMSCs occurred only when COX-2 activity was absent in both mesenchymal and hematopoietic cells. Using co-cultures and conditioned media (CM) from bone marrow macrophages (BMMs), we show that the inhibition of PTH-stimulated OB differentiation was mediated by a factor or factors secreted by hematopoietic cells committed to the OC lineage in response to COX-2 produced PGs or to added PGE_2. This study reveals a new role for COX-2 and PGE_2 in regulating PTH-stimulated responses in bone and a new example of regulation of OB differentiation by OCs.

Materials and methods

Materials

PGE_2, NS398, MRE-269 (prostaglandin IP receptor agonist), dinoprost ($PGF_{2\alpha}$ receptor agonist) and all other prostanoids used were from Cayman Chemical Company (Ann Arbor, MI). Recombinant mouse macrophage-colony stimulating factor (M-CSF), osteoprotegerin (OPG)/Fc-chimera and RANKL were from R&D systems (Minneapolis, MN). Bovine PTH (bPTH; 1–34) and all other chemicals were from Sigma (St. Louis, MO), unless otherwise noted.

Animals

Mice with disruption of *Ptgs2*, which produce no functional COX-2 protein, called *Cox-2* knockout (KO) mice, in a C57BL/6, 129SV background were the gift of Scott Morham [27]. *Ptger2* and *Ptger4* KO mice in C57BL/6, 129 backgrounds were gifts from Richard and Matthew Breyer [28,29]. All KO mice were backcrossed more than 16 generations into the CD-1 (outbred) background. Breeding colonies were refreshed twice a year by regenerating maintenance colonies from mice heterozygous for the deleted or disrupted gene mated with WT mice from Jackson Laboratory (Bar Harbor, ME). For experiments, *Cox-2* KO mice were bred by KO × KO mating, and *Ptger2* and *Ptger4* KO mice were bred by heterozygous × heterozygous mating. Genotyping protocols were as described previously [30–32]. All animal studies were conducted in accordance to the approved protocols by the Animal Care and Use Committee of the University of Connecticut Health Center.

Cell cultures

All cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Basic medium was α-MEM (Invitrogen, Carlsbad, CA), 10% heat inactivated fetal calf serum (HIFCS), 100 U/ml penicillin, and 50 μg/ml streptomycin. Vehicles for the various treatments were as follows: 0.1% ethanol for PGE_2, all other prostanoid receptor agonists, and NS398; 0.1% bovine serum albumin (BSA) in 1× phosphate buffered saline (PBS) for RANKL, M-CSF and OPG; dimethyl sulfoxide for isobutyl methyl xanthine (IBMX); and 0.001 N hydrochloric acid-acidified 0.1% BSA in 1× PBS for PTH.

To make bone marrow stromal cell (BMSC) cultures, whole marrow flushed from tibiae and femora of 6–8 week old mice, plated at 10^6 nucleated cells/well in 6-well tissue culture dishes and cultured in OB differentiation medium from the time of plating onward. Differentiation medium consisted of basic medium plus 50 μg/ml phosphoascorbate (Wako Pure Chemical Industry, Osaka, Japan). To study mineralization, 8 mM of β-glycerophosphate was added on day 7. Media were changed every 3–4 days. Unless specified, all agents were added from the beginning of culture and with each medium change. To make primary osteoblast (POB) cultures, calvariae from 5 to 6 neonatal mice were dissected free of sutures, minced, washed with 1× PBS and digested with 0.5 mg/ml of collagenase P (Roche Diagnostics, Indianapolis, IN) in a solution of 1 ml 0.25% trypsin/EDTA and 4 ml PBS at 37 °C. Four digests were performed for 10 min each and a final digest for 90 min. Digests 2–5 were pooled and plated at 4×10^4 cells/well in 6-well dishes and cultured in differentiation media. To make bone marrow macrophage (BMM) cultures, we followed the protocols of R. Faccio http://www.orthoresearch.wustl.edu/content/Laboratories/2978/Roberta-Faccio/Faccio-Lab/Protocols.aspx. Briefly, 10^7 nucleated bone marrow cells/well were plated in 150 mm Petri dishes (Fisher Scientific, Pittsburgh, PA) in basic medium plus 100 ng/ml M-CSF and expanded twice, each for three days, before being used for co-culture or conditioned media experiments.

Co-culture and conditioned media (CM) experiments

For co-culture of BMMs and POBs, POBs were plated at 4×10^4 with 4×10^5 BMMs (1:10 ratio) per well in 6-well tissue culture dishes and cultured in OB differentiation medium. For co-culture of BMMs and BMSCs, BMMs were plated at 1:3 with BMSCs and cultured in OB differentiation medium. To obtain CM, BMMs were re-plated at 6×10^4 cells/well in 12 well tissue culture dishes in basic medium plus 30 ng/ml M-CSF with/without RANKL (30 ng/ml). CM were collected, pooled and centrifuged at 800 rpm for 5 min at 4 °C to get rid of debris and kept frozen until use. For differentiation studies with CM, freshly isolated POBs were plated at 4×10^4/well in 6-well dishes and cultured in 3 parts of CM and 1 part OB differentiation media with 50 ng/ml of OPG to block RANKL–RANK interactions that might generate osteoclasts.

Real-time (quantitative) PCR analysis

Total RNA was extracted with Trizol (Invitrogen) following manufacturer's instructions. 2–5 μg of total RNA was DNase treated (Ambion, Inc., Austin, TX) and converted to cDNA by the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). PCR was performed in 96-well plates. Both Assays-on-Demand Gene Expression Taqman primers (Applied Biosystems) and validated Syber Green primers (http://pga.mgh.harvard.edu/primerbank) were used for PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin served as endogenous control. All primers were checked for equal efficiency over a range of target gene concentrations. Each sample was amplified in duplicate. PCR reaction mixture was run in Applied Biosystems Prism 7300 Sequence Detection System instrument utilizing universal thermal cycling parameters. Data analysis was done using relative quantification (RQ, ΔΔCt) or the relative standard curve method.

OB and osteoclast-like cell (OCL) staining

For alkaline phosphatase (ALP) staining, cells were fixed and stained with an alkaline phosphatase kit (Sigma) using the manufacturer's instructions. Dishes were air dried and scanned into the computer. To assess mineralization, cells were washed with PBS, fixed in 100% V/V methanol on ice for 30 min and stained with 40 mM alizarin red-S pH 4.2 for 10 min at room temperature. Dishes were washed with water, air dried and scanned into the computer. For tartrate resistant acid phosphatase (TRAP) staining, cells were fixed with 2.5% glutaraldehyde in PBS for 30 min at room temperature and stained by the Leukocyte Acid Phosphatase Kit (Sigma) following company's instructions.

Oil red O staining

BMSCs were cultured for 14 days under similar conditions used for OB differentiation but without phosphoascorbate and β-glycerophosphate in the culture medium. Instead, 1 μM of insulin was added to the medium on day 7 to induce formation of fat bodies. For staining, cells were washed twice with 1× PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, rinsed with water and then incubated with oil red O working solution (3 parts to 2 parts water) for 1 h at room temperature. Dishes were washed with water, air dried and scanned into the computer.

Prostaglandin (PG)E_2 assay

Media were removed from cultured cells and frozen until assay. PGE_2 accumulation was measured using an enzyme immunoassay (correlate-EIA™) kit following the manufacturer's instructions (Assay Designs, Ann Arbor, MI).

Intracellular cAMP measurement

Confluent POBs were treated with 3 parts CM and 1 part of OB differentiation medium containing 0.5 mM isobutyl methyl xanthine (IBMX) 1 h prior to adding PTH or PGE_2 for 20 min. Cells were scraped off in 400 μl/well of ice-cold ethanol. The ethanolic cell suspension was collected in tubes and centrifuged at 1500 ×g for 10 min at 4 °C. Supernatants were collected and evaporated to dryness using a lyophilizer. cAMP was measured using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Statistics

All data are presented as means ± SEM. Analysis was performed using SigmaPlot 11.0 (Systat Software, Inc.). Experiments involving several genotypes (or combinations of genotypes in co-cultures) and treatments were examined by two-way ANOVA, followed by post hoc Bonferroni pairwise multiple comparison. If data were not normally distributed, they were transformed (log 10) before ANOVA. Comparison of multiple treatments to a single control was examined by one-way ANOVA, followed by Bonferroni pairwise multiple comparison. If these data were not normally distributed, they were examined by one-way ANOVA on ranks, followed by Dunn's Test for all pairwise multiple comparisons.

Results

Endogenous PGs suppressed PTH-stimulated OB differentiation in BMSC cultures

To study effects of endogenous PGs on PTH-stimulated OB differentiation, we used BMSCs from WT and *Cox-2* KO mice. Despite the constitutive expression of *Cox-1*, very little PGE_2 is measurable in the media of *Cox-2* KO BMSC cultures [14,33]. It is expected that there will be “basal” production of PGE_2 in WT BMSC cultures because fresh serum stimulates *Cox-2* expression [34]. Because PGE_2 can stimulate OB differentiation, this basal production often leads to increased OB differentiation in vehicle-treated WT compared to KO or NSAID-treated WT cultures, as seen here (*e.g.*, Figs. 1A–E). PTH is expected to further induce *Cox-2* expression and PGE_2 production in these cultures [12,13].

BMSCs were cultured with PTH (10 nM) added at plating of cells and with each media change. This protocol should provide continuous exposure to PTH because PTH has been shown to be stable in culture up to 72 h between medium changes [35]. As we showed previously [26], PTH stimulated OB differentiation in *Cox-2* KO, but not WT, BMSC cultures. PTH stimulated marked increases in *Alp* and *Osteocalcin* mRNA (Figs. 1A,B) and alizarin red staining (data not shown) in KO cultures, but not in WT cultures. In WT cultures, PTH decreased, or tended to decrease, markers of OB differentiation relative to vehicle treatment. The stimulatory effect of PTH in *Cox-2* KO cultures was seen by day 7 of culture and was maintained throughout 3 weeks of culture (Fig. 1A).

To determine if the inhibitory effect of COX-2 was due to COX-2 activity, we examined treatment with a selective inhibitor of COX-2 activity, NS398. NS398 restored the ability of PTH to stimulate *Alp* and *Osteocalcin* mRNA expression and alizarin red staining in WT cultures,

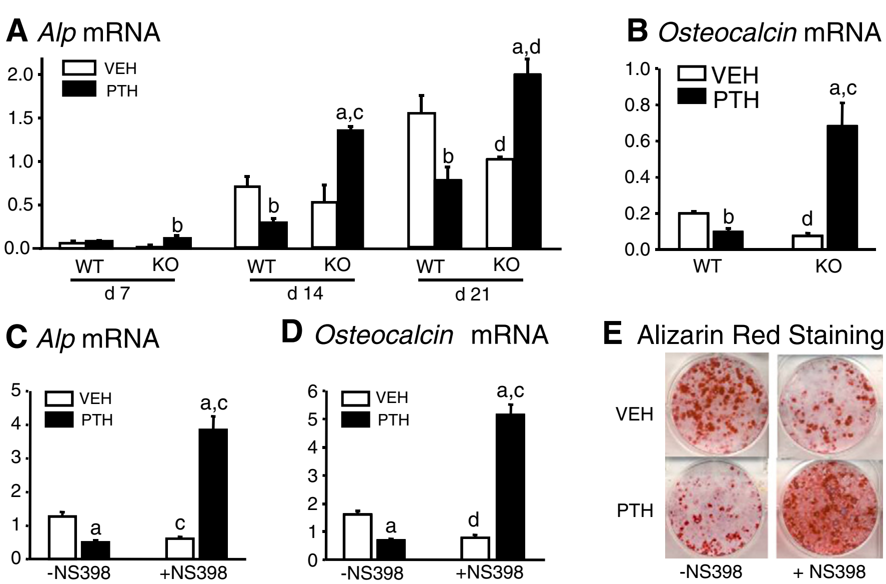

Fig. 1. Effects of COX-2 expression or activity on PTH-stimulated OB differentiation in bone marrow stromal cell (BMSC) cultures. BMSCs from WT and *Cox-2* knockout (KO) mice were plated in osteogenic media and treated with vehicle (VEH) or PTH (10 nM) begun at the time of plating and given with each media change. Gene expression was measured by real time PCR (qPCR). (A) Time course for PTH effects on *Alp* mRNA expression in WT and KO cells. (B) *Osteocalcin* mRNA expression at day 21 of the culture shown in (A). Effects of NS398 (0.1 μM), a selective inhibitor of COX-2 activity, on PTH-stimulated *Alp* mRNA (C), *Osteocalcin* mRNA (D) and mineralization (E) in WT BMSCs, measured at 14 days of culture. Bars are means ± SEM for n = 3 wells of cells. [a]Significant effect of treatment relative to vehicle, $p < 0.01$, [b]$p < 0.05$. [c]Significant effect of genotype, $p < 0.01$, [d]$p < 0.05$.

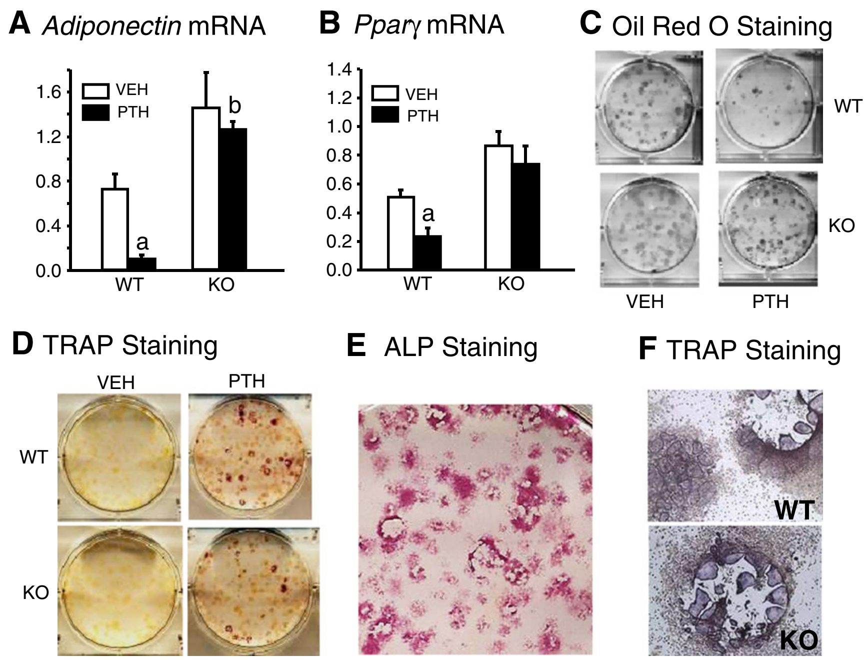

Fig. 2. PTH regulation of adipogenesis and osteoclast-like cell (OCL) formation in BMSCs from WT and *Cox-2* KO mice. BMSCs were cultured in osteogenic media, except as noted, and treated with vehicle (VEH) or PTH (10 nM). mRNA expression was measured by qPCR. (A) *Adiponectin* and (B) *Pparγ* mRNA measured at 14 days of culture. Data are from same experiment as shown in Figs. 1 (A,B). Bars are means ± SEM for n = 3. [a]Significant effect of treatment relative to vehicle, $p < 0.01$. [b]Significant effect of genotype, $p < 0.01$. (C) Oil red O staining in BMSCs cultured for 14 days. Cells were cultured without phosphoascorbate and β-glycerophosphate. Insulin (1 μM) was added to the medium on day 7. (D) Tartrate resistant acid phosphatase (TRAP) staining in BMSCs at day 7 of culture. (E) Alkaline phosphatase (ALP) staining of colonies in PTH-treated *Cox-2* KO BMSCs at day 9 of culture. (F) TRAP staining (40× magnification) at day 7.

confirming that the inhibitory effects were due to PG production (Figs. 1C–E).

Because there may be reciprocal effects between OB and adipocyte differentiation [36,37] and because PTH can regulate adipocyte differentiation [35], we examined expression of *Adiponectin*, a marker of adipocytes, and *Pparγ*, a transcription factor that may be important not only for stimulating adipogenesis but also for suppressing osteogenesis [38]. PTH inhibited both *Adiponectin* and *Pparγ* expression on day 14 of culture in WT, but not *Cox-2* KO, cultures (Figs. 2A,B). Similar patterns were seen on day 21 (data not shown). Oil red O staining for mature adipocytes was consistent with the gene expression (Fig. 2C). Hence, differentiation of both OBs and adipocytes in these cultures was inhibited by endogenous PGs.

BMSC cultures differ from the marrow cultures used for studying OC differentiation in that they are plated at lower density and have phosphoascorbate in the media. PTH stimulated formation of osteoclast-like cells (OCLs), defined as tartrate resistant acid phosphatase (TRAP) multinucleated cells, during the first week of culture in both WT and *Cox-2* KO BMSCs. OCLs were seen at days 4–5 of culture and were abundant by day 7, resulting in the appearance of "empty" areas in the center of ALP stained colonies (Figs. 2D–F). No OCLs were formed in control cultures (Fig. 2D). OCLs had largely disappeared by days 12–14 (data not shown). It was not possible to quantify OCL number in these cultures since most were covered by a canopy of cells. Although there appeared grossly to be little difference in TRAP staining between WT and KO cells, these observations raised the possibility that differences

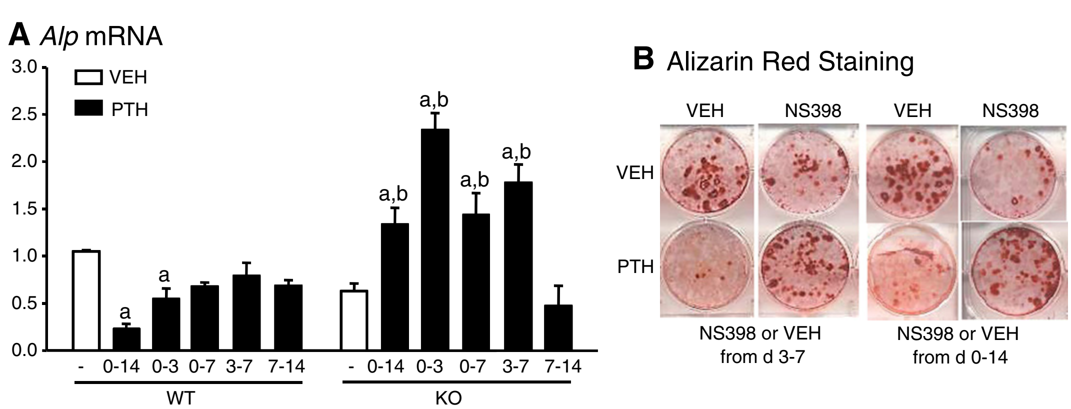

Fig. 3. Window of time for PTH stimulation of OB differentiation in BMSCs. BMSCs from WT and *Cox-2* KO mice were plated in osteogenic media and treated with vehicle (VEH) or PTH (10 nM) begun at the time of plating and given with each media change. Gene expression was measured by qPCR. (A) Comparison of the *Alp* mRNA response to different periods of treatment with PTH. BMSCs were given PTH for the days indicated on x-axis. All cultures were extracted for RNA after 14 days. Bars are means ± SEM for n = 3 wells of cells. [a]Significant effect of PTH, $p < 0.01$. [b]Significant effect of genotype, $p < 0.01$. (B) Comparison of the mineralization response to PTH in WT BMSCs given NS398 for 3–7 days versus 0–14 days. All cultures were stained for alizarin red after 14 days of culture.

in PTH-simulated OB differentiation between WT and KO cultures might be due to space-occupying OCLs.

To determine the window of time during which PTH needed to be present to stimulate OB differentiation, we cultured BMSCs for different periods of time with PTH and measured *Alp* mRNA at day 14 of culture. When PTH was given to *Cox-2* KO BMSCs from days 0–3, 3–7 or 0–7 of culture, it increased *Alp* mRNA (Fig. 3A). However, when PTH was not started until day 7 of culture, it did not increase OB differentiation. PTH did not stimulate *Alp* mRNA expression in WT BMSCs when given for any period of time. As further confirmation that PTH acted during the first week of culture to stimulate OB differentiation, we treated WT BMSCs with NS398 from days 3 to 7 or from days 0 to 14 and measured mineralization on day 14. PTH stimulated mineralization to a similar extent in both cases (Fig. 3B).

Because the window for PTH stimulation of OB differentiation in *Cox*-2 KO cultures was early in culture and because PGs cause PTH to decrease both OB and adipocyte differentiation, it is possible that PGs are modulating the actions of PTH on MSCs, which are likely to be available only early in culture.

Suppression of PTH-stimulated OB differentiation required both PGs and hematopoietic cells

Because OCLs formed early in BMSC cultures, beginning during the window of time for the stimulatory effects of PTH, we postulated that OC lineage cells might play a role in the inhibitory effects of PGs. If so, the inhibitory effect should not be seen in primary osteoblasts (POBs). However, in our previous study, we also observed an inhibitory effect of PGs on PTH-stimulated OB differentiation in POB cultures [26]. When we examined our POB cultures for the ability to form OCLs, we found that both PTH, which increases RANKL mRNA expression in POBs, and exogenous RANKL induced formation of cells that stained for TRAP in these cultures (Fig. 4A). If the minced calvarial pieces were carefully washed multiple times to eliminate marrow, many fewer TRAP staining cells were seen (Fig. 4A). With the increased washing of calvarial pieces, we found that PTH stimulated OB differentiation in WT POBs (Fig. 4B) and that NS398 had no effect on PTH-stimulated OB differentiation (Fig. 4C).

On the assumption that PGE_2 might be the PG mediating the inhibitory effects of COX-2, we examined the effects of adding PGE_2 to PTH (Fig. 4D). (We continued to use either *Cox-2* KO POBs or treat with NS398 because chronic exposure to PGE_2 in the media might down regulate responses to added PGE_2.) PTH or PGE_2 alone stimulated *Alp* mRNA in POBs at 14 days of culture, but the combination of PTH and PGE_2 had no greater effect than either agent alone, suggesting that some inhibition remained (Fig. 4D). However, treatment of POBs with PTH, PGE_2 and the combination for 15 min had an additive effect on cAMP production (Fig. 4E), the pathway through which both agents are supposed to produce anabolic effects. Because we had previously observed that the combination of PGE_2 and PTH had additive or greater effects on OCL formation in bone marrow cultures [31], we treated cultures with OPG, which interrupts the RANK–RANKL interaction. In the presence of OPG, the combination of PTH and PGE_2 had additive effects on PTH-stimulated *Osteocalcin* mRNA at 14 days (Fig. 4F).

These data suggest that RANKL-stimulated hematopoietic cells were necessary for suppression of PTH-stimulated OB differentiation. In addition, the data indicate that PGE_2 itself was not the factor that acted on POBs to inhibit PTH-stimulated OB differentiation.

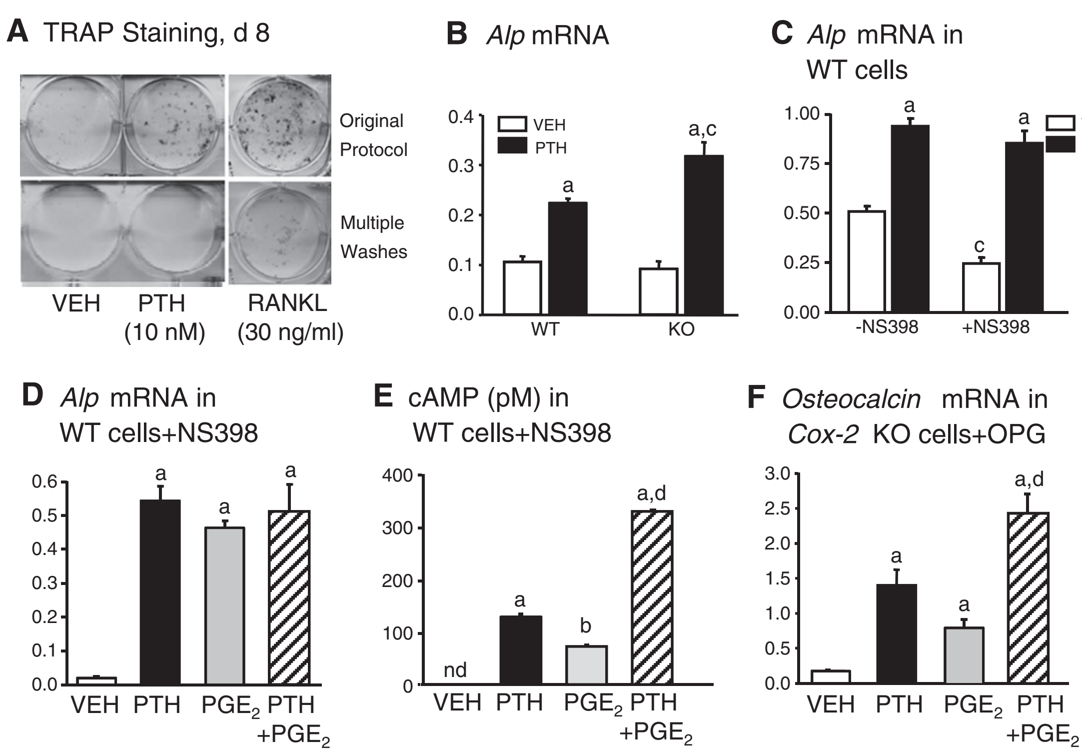

Fig. 4. Effects of COX-2 expression or activity on PTH-stimulated differentiation in primary osteoblast (POB) cultures. POBs from WT and *Cox-2* KO mice were plated in osteogenic media and treated with vehicle (VEH), PTH (10 nM), or other agents begun at the time of plating and given with each media change. Gene expression was measured by qPCR. (A) Stimulation of TRAP-staining cell formation in POBs cultured from WT mice. Cultures were treated with PTH or RANKL (30 ng/ml) for 8 days. (B) *Alp* mRNA expression in WT and *Cox-2* KO POBs treated for 14 days. (C) Effect of NS398 (1 μM), an inhibitor of COX-2 activity, on *Alp* mRNA expression in WT POBs at 14 days. (D) Comparison of PTH or PGE_2 (1 μM) or their combination, PTH+PGE_2, all in the presence of NS398 (1 μM), on *Alp* mRNA expression at 14 days. (E) Measurement of intracellular cAMP in WT POB cultures in the presence of NS398 (1 μM). Cells were treated with the same agonists as in (D) for 15 min in the presence of IBMX (0.5 mM). (F) Treatment of *Cox-2* KO POBs with PTH, PGE_2 (10 nM), or PTH + PGE_2 in the presence of osteoprotegerin (OPG, 50 ng/ml), an inhibitor of RANKL-mediated osteoclast formation. *Osteocalcin* mRNA was measured at 21 days. Bars are means ± SEM for n = 3 wells of cells. [a]Significant effect of treatment, $p < 0.01$; [b]$p < 0.05$. [c]Significant effect of genotype, $p < 0.05$. [d]Significant effect of PTH + PGE_2 relative to either treatment alone, $p < 0.01$. Nd = below limits of assay.

Bone marrow macrophages (BMMs) expressing COX-2 were sufficient to prevent the PTH-stimulated OB differentiation

The addition of WT BMMs to *Cox-2* KO BMSCs blocked the PTH-stimulation of OB differentiation (Fig. 5A). When *Cox-2* KO POBs were co-cultured with BMMs from WT or *Cox-2* KO mice, the presence of WT BMMs, but not KO BMMs, prevented the PTH-stimulated increase in OB mineralization (Fig. 5B). To confirm a role for cells committed to the OC lineage in mediating the inhibitory effect of PGs, we treated BMSCs with OPG. When OPG was present, PTH stimulated OB differentiation in WT as well as *Cox-2* KO BMSCs (Figs. 5C–E). Although OPG is reported to have direct effects on OB differentiation [39], we did not see effects of OPG alone on OB differentiation. We considered the possibility that OPG might block inhibitory effects by suppressing PG production in these cultures. There was a reduction, not statistically significant, in PTH-stimulated medium PGE_2 accumulation in the presence of OPG from 7.3 ± 0.4 to 4.4 ± 1.6 nM, which, as will be discussed below, should not have prevented the inhibitory effects. These results are consistent with the previous data suggesting that the cells mediating the inhibition of PTH-stimulated OB differentiation are committed to the OC lineage.

Although OBs are generally assumed to be the major source of PGs in bone, these co-culture results suggested that WT BMMs produced sufficient PGs to mediate the inhibitory effects. To examine the relative roles of OB and OC lineage cells in producing PGE_2 in these cultures, we measured medium PGE_2 accumulation in co-cultures of POBs and BMMs from WT and *Cox-2* KO mice and compared with OB differentiation measured by *Osteocalcin* mRNA (Table 1). As expected, when KO POBs were co-cultured with KO BMMs, medium PGE_2 was undetectable in vehicle or PTH-stimulated cultures [31,33]. WT BMMs (plated at 10:1 ratio with POBs) made more PGE_2 under basal conditions than WT POBs. The basal level of PGE_2 production by POBs was likely due to the serum induction of COX-2 [34]. PTH stimulated PGE_2 production 2- to 3-fold in co-cultures with WT POBs but had little effect in cultures with KO POBs, consistent with the expected absence of PTH receptors on BMMs. The small increase in PGE_2 in the WT BMM, KO POB co-culture might be due to PTH-stimulated RANKL expression in the POBs, which subsequently induced COX-2 in BMMs [40].

In vehicle-treated cultures, the *Osteocalcin* levels decreased as PGE_2 levels decreased (Table 1). PTH-stimulated *Osteocalcin* mRNA expression was increased 20-fold relative to vehicle treatment in KO BMM-KO POB cultures, which had no detectable PGE_2 production. In all other combinations, which contained WT POBs or WT BMMs and did produce measurable PGE_2, PTH-stimulated *Osteocalcin* expression

Table 1
Medium PGE_2 and *Osteocalcin* mRNA expression in co-cultured POBs and BMMs from WT and COX-2 KO mice treated with vehicle or PTH (10 nM).

	WT BMMs		KO BMMs	
	WT POBs	KO POBs	WT POBs	KO POBs
(1) PGE_2 (nM) accumulated in media during first week of culture				
Vehicle	6.8 ± 0.1	6.4 ± 0.2	$3.8 \pm 0.1^{a,b}$	nd
PTH	14.2 ± 0.4^{c}	$7.2 \pm 0.2^{a,c}$	$11.3 \pm 0.2^{a,b,c}$	nd
(2) Osteocalcin mRNA at day 14 of culture				
Vehicle	1.71 ± 0.13^{d}	1.33 ± 0.07^{d}	1.07 ± 0.12^{d}	0.55 ± 0.05
PTH	$0.12 \pm 0.01^{c,d}$	$0.09 \pm 0.01^{c,d}$	$0.19 \pm 0.02^{c,d}$	10.9 ± 1.24^{c}

(1) PGE_2 data are means ± SEM for n = 3. Nd is not detectable. Media were collected at time of media change (day 3 and day 7) and equal aliquots pooled for measurement. (2) *Osteocalcin* data are means ± SEM for n = 4. For both sets, data were log 10 transformed before two-way ANOVA analysis to achieve normal distribution.
[a] Significantly different from WT BMMs + WT POBs, $p < 0.01$.
[b] Significantly different from WT BMMs + KO POBs, $p < 0.01$.
[c] Significant effect of PTH, $p < 0.01$, $p < 0.05$.
[d] Significantly different from KO BMMs + KO POBs, $p < 0.01$.

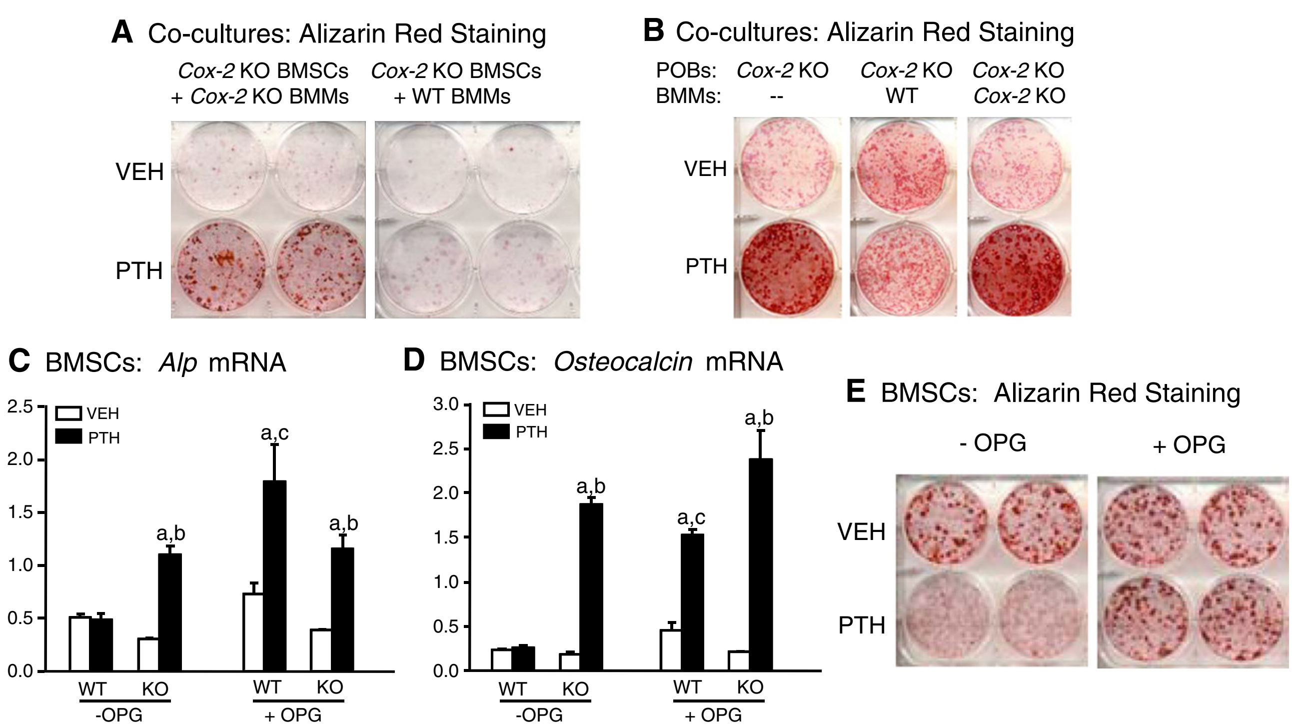

Fig. 5. Effects of bone marrow macrophages (BMMs) on PTH-stimulated OB differentiation. BMSCs or POBs from WT and *Cox-2* KO mice were cultured alone or with bone marrow macrophages (BMMs). All cultures were in osteogenic media and treated with vehicle (VEH), PTH (10 nM), or other agents began at the time of plating and given with each media change. Gene expression was measured by qPCR. (A) Alizarin red staining at 14 days in *Cox-2* KO BMSCs co-cultured with BMMs from KO and WT mice. BMMs were plated at a ratio of 1:3 to BMSCs. (B) Alizarin red staining at 14 days in *Cox-2* KO POBs co-cultured with BMMs from WT and KO mice. POBs were plated at a ratio of 1:10 to BMMs. (C) *Alp* and (D) *Osteocalcin* mRNA expression in WT and *Cox-2* KO BMSCs at 14 days following treatment with PTH, plus/minus osteoprotegerin (OPG, 30 ng/ml), which interferes with RANKL-RANK binding. (E) Alizarin red staining at 14 days of culture in WT BMSCs treated with PTH plus/minus OPG (50 ng/ml). Bars are means and SEM for n = 3 wells of cells. [a]Significant effect of PTH, $p < 0.01$. [b]Significant effect of genotype, $p < 0.01$. [c]Significant effect of OPG, $p < 0.01$.

was inhibited relative to the KO-KO combination. Hence, either POBs or BMMs expressing COX-2 were sufficient to prevent the PTH-stimulated OB differentiation in this culture system.

Exogenous PGE_2 suppressed PTH-stimulated OB differentiation in BMSCs

In many of our experiments in BMSC cultures (Figs. 1,3) or in cultures with both POBs and BMMs (Table 1), but not in POBs cultured alone (Fig. 5), PTH given in the presence of COX-2 expression resulted in decreased *Alp* or *Osteocalcin* expression relative to vehicle-treated cultures. Since some of the OB differentiation in vehicle-treated cultures is explainable by the serum induction of COX-2 expression and endogenous PGE_2 production (Table 1) [34], this observation suggests that, in the presence of BMMs, the stimulatory effect of endogenous PGE_2 on OB differentiation was suppressed in the presence of PTH.

To look at this possibility more directly, we treated BMSC cultures with PTH (10 nM), PGE_2 (10 nM) and the combination (Fig. 6A). PGE_2 stimulated *Bone sialoprotein* (*Bsp*) mRNA at 14 days in both WT and *Cox*-2 KO BMSCs. (The small but significant increase in the effects of PGE_2 in KO cells has been seen before and may be due to down regulation of PGE_2 receptors due to chronic exposure to endogenous PGE_2 in WT cultures). Although both PTH and PGE_2 individually stimulated *Bsp* mRNA expression in KO cultures, the combination of PTH and PGE_2 had no stimulatory effect.

To better understand the dose range over which these effects occurred, we treated *Cox-2* KO BMSCs with PTH (10 nM) ± PGE_2 (0.1 nM to 0.1 μM) for 14 days (Fig. 6B). PTH and all doses of PGE_2 alone increased *Alp* mRNA relative to vehicle, but PTH combined with PGE_2, at any dose, resulted in decreased *Alp* mRNA expression relative to either PTH or PGE_2 alone. Similarly, the combination of a single dose of PGE_2 (10 nM) with several doses of PTH (0.1 nM to 10 nM) decreased *Alp* mRNA expression relative to PGE_2 or PTH alone (Fig. 6C). To examine a role for BMMs in the inhibition of OB differentiation by the combination of PTH and PGE_2, we examined the effects of OPG (Fig. 6D). In the presence of OPG, the combination of PTH and PGE_2 had additive stimulatory effects on *Osteocalcin* mRNA.

Other PGs could be involved in the inhibitory effect of COX-2. To screen for some other likely candidates, we treated *Cox-2* KO BMSCs with PGE_2 and compared with other PG receptor agonists, all at 0.1 μM (Fig. 6E). Because PGI_2 is unstable, we used MRE-269, a stable IP receptor agonist. For $PGF_{2\alpha}$, we used dinoprost, an FP receptor agonist. All cultures were with *Cox-2* KO cells because PGs can induce COX-2 expression and make PGE_2, which could confound the comparison [41]. PGE_2 was the only prostanoid that stimulated *Osteocalcin* mRNA, and the only prostanoid that resulted in loss of the stimulatory effect when added to PTH.

These data on exogenous PGE_2, along with the previous data on endogenous PGs, can be summarized as follows (Table 2). The inhibition of

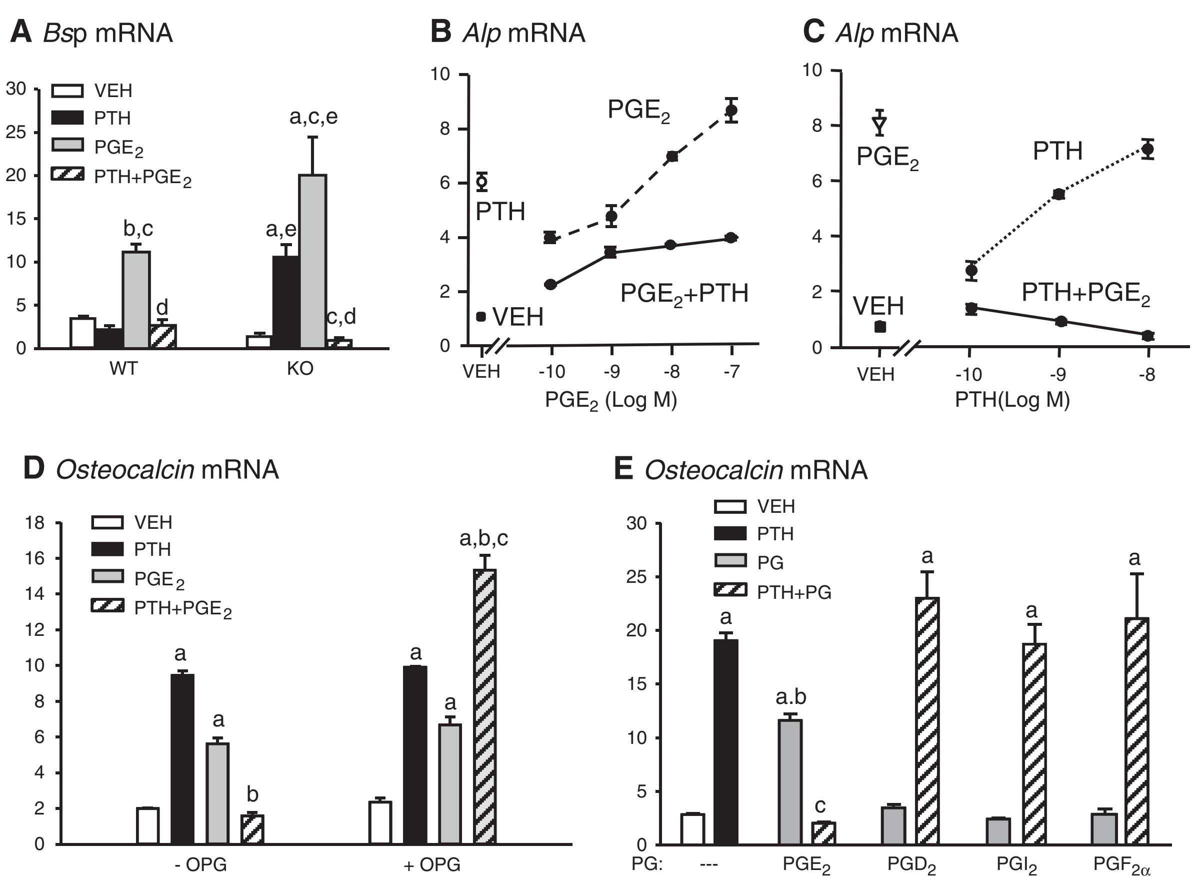

Fig. 6. Effects of exogenous PGs on PTH-stimulated differentiation in BMSCs. BMSCs were treated with vehicle (VEH), PTH, PG or the combination of PTH and PG at plating and at every media change. (A) *Bone sialoprotein* (*Bsp*) mRNA expression in WT and *Cox-2* KO BMSCs treated for 14 days with PTH (10 nM) and PGE_2 (10 nM). Bars are means ± SEM for n = 3 wells. [a]Significant effect of treatment relative to vehicle, $p < 0.01$, [b]$p < 0.05$. [c]Significantly different from PTH alone, $p < 0.01$. [d]Significantly different from PGE_2 alone, $p < 0.01$. [e]Significant effect of genotype, $p < 0.01$. (B) *Alp* mRNA in *Cox-2* KO BMSCs at 14 days of culture: effect of adding varying doses of PGE_2 to PTH (10 nM). Symbols are means ± SEM for n = 3 wells. PTH and all doses of PGE_2 alone increased Alp mRNA relative to vehicle ($p < 0.01$). The combination of PTH and PGE_2 at all doses reduced *Alp* mRNA relative to either agent alone ($p < 0.01$). (C) *Alp* mRNA in *Cox-2* KO BMSCs at 14 days of culture: effect of adding varying doses of PTH to PGE_2 (10 nM). Symbols are means ± SEM for n = 3 wells. PGE_2 and all doses of PTH increased *Alp* mRNA relative to vehicle ($p < 0.01$). The combination of PTH and PGE_2 at all doses reduced *Alp* mRNA relative to either agent alone ($p < 0.01$). (D) *Osteocalcin* mRNA in *Cox-2* KO BMSCs at 14 days of culture: effect of OPG (50 ng/ml). Symbols are means ± SEM for n = 3 wells. [a]Significant effect of treatment relative to vehicle, $p < 0.01$. [b]Significantly different from PTH or PGE_2 alone, $p < 0.01$. [c]Significant effect of OPG, $p < 0.01$. (E) *Osteocalcin* mRNA in *Cox-2* KO BMSCs at 14 days of culture: effect of different PGs (all at 0.1 μM). Symbols are means ± SEM for n = 3 wells. [a]Significant effect of treatment relative to vehicle, $p < 0.01$. [b]Significantly different from all other PGs alone, $p < 0.01$. [c]Significantly different from PTH or PGE_2 alone and all other combinations of PTH and PG, $p < 0.01$.

Table 2
Summary of effects of PTH, COX-2/PGE_2 and BMMs on OB differentiation.

Treatments	Cells			
	OBs with BMMs		OBs without BMMs	
	COX-2 in OB or BMM	No COX-2	COX-2	No COX-2
PTH	−	+	+	+
PGE_2	+	+	+	+
PTH + PGE_2	−	−	++	++

PTH-stimulated OB differentiation was only seen in the presence of both BMMs and endogenous or exogenous PGs. In the absence of BMMs, there was no inhibitory effect of COX-2 or PGE_2, and PTH and PGE_2 were additive. In the presence of BMMs, treatment with the combination of PTH and PGE_2, each of which was stimulatory alone, produced no stimulatory effect.

Inhibition of PTH-stimulated OB differentiation required expression of EP4 receptors (EP4) on BMMs

The need for BMMs to be present in order to see inhibition of PTH effects suggests that PGs are acting on BMMs to cause the inhibition. As indicated by the studies above, PGE_2 is a likely candidate for the PG involved. The effects of PGE_2 in bone have been most often associated with cAMP production and protein kinase A (PKA) activation, suggesting an important role for the PGE_2 receptors EP2 and EP4, which are both coupled to $G\alpha_s$. Both EP2 and EP4 are reported to be expressed by bone marrow macrophage OC precursors [42]. To examine the roles of these receptors, BMSCs from WT and *Ptger2* or *Ptger4* KO mice were cultured with PTH (Figs. 7A,B). PTH stimulated OB differentiation in *Ptger4* KO cultures but inhibited in WT and *Ptger2* KO cultures. For comparison, we treated these cultures with PGE_2. PGE_2 stimulated *Osteocalcin* expression in both WT and *Ptger2* KO BMSC (Figs. 7A,B). As expected from previous experiments, which showed a major role for EP4 in the osteogenic effects of PGE_2 [43,44], deletion of *Ptger4* greatly reduced PGE_2-mediated OB differentiation.

To determine if EP4 on BMMs was necessary for the suppression of PTH effects, we co-cultured *Cox-2* KO POBs with BMMs from WT, *Cox-2* KO and *Ptger4* KO mice (Fig. 7C). As expected, PTH stimulated *Osteocalcin* expression in POBs cultured without BMMs and in POBs co-cultured with *Cox-2* KO BMMs but not with WT BMMs. There was no inhibition of PTH-stimulated *Osteocalcin* expression in POBs co-cultured with *Ptger4* KO BMMs. To rule out the possibility that the effect of *Ptger4* deletion was due to preventing formation of OC precursors, we compared the co-cultures for TRAP staining. There was no increase in TRAP staining with PTH in cultures without BMMs. PTH increased TRAP similarly in all the other co-cultures (Fig. 7D). Hence, *Ptger4* in BMMs was required for the inhibitory effects of PGs on PTH-stimulated OB differentiation.

The inhibitory effect was transferred by conditioned media (CM) from RANKL-stimulated BMMs

To determine if the inhibition was mediated by cell–cell contact or by secretion of a soluble factor, POBs were co-cultured with CM

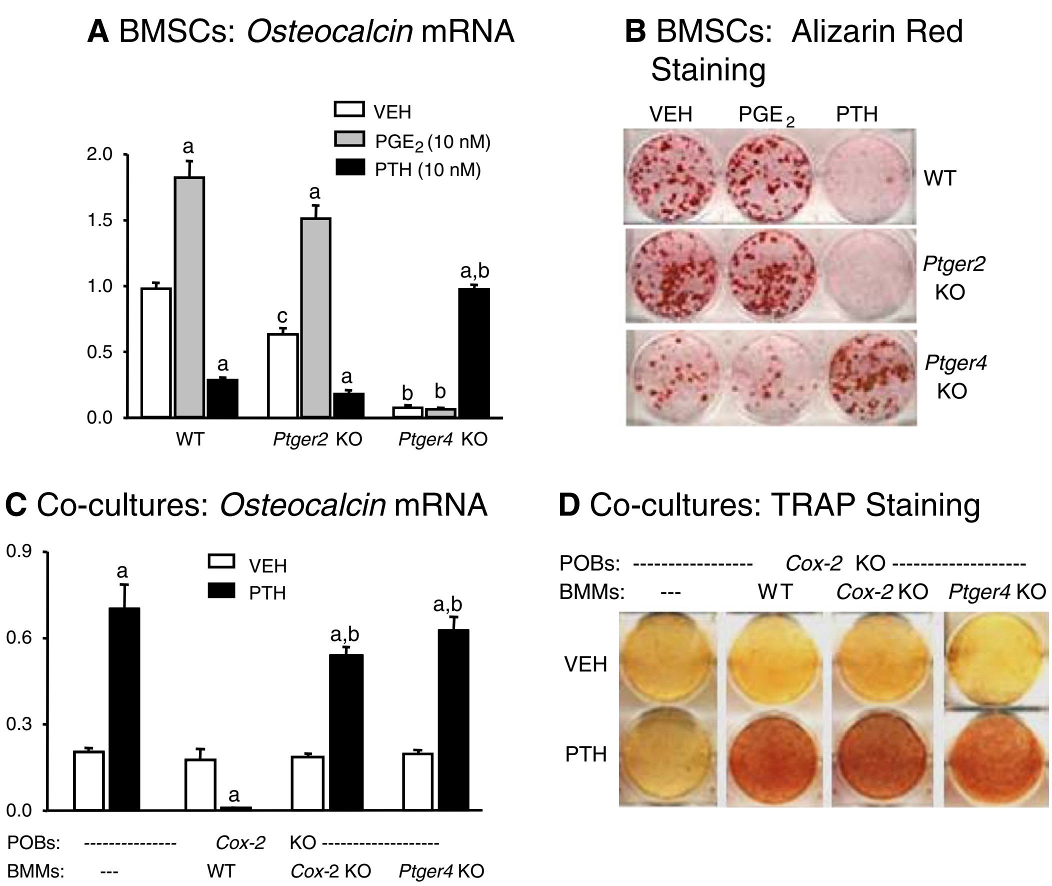

Fig. 7. Effects of deleting *Ptger2* and *Ptger4* on PTH-stimulated OB differentiation. All cells were cultured in osteogenic media. Treatments were begun at the time of plating and given with each media change. Gene expression was measured by qPCR. (A) *Osteocalcin* mRNA and (B) alizarin red staining in WT, *Ptger2* KO and *Ptger4* KO BMSCs at day 14 of culture. BMSCs were treated with vehicle (VEH), PTH (10 nM) and PGE_2 (10 nM). (C) *Osteocalcin* mRNA in *Cox-2* KO POBs co-cultured with BMMs from WT, *Cox-2* KO and *Ptger4* KO mice for 14 days. POBs were plated at a ratio of 1:10 to BMMs. (D) TRAP staining in POB and BMM co-cultures at day 8. Bars are means ± SEM for n = 3. [a]Significant effect of treatment, $p < 0.01$. [b]Significant effect of genotype compared to WT, $p < 0.01$, [c]$p < 0.05$.

collected from WT and *Cox-2* KO BMMs. *Cox-2* KO POBs were used in all experiments, and *Alp* or *Osteocalcin* mRNA was measured after 14 days of culture. Because RANKL was added to most BMM cultures before obtaining the CM, all POB cultures were done in the presence of OPG to prevent OCL formation.

In the first experiment, CM was collected from BMMs expanded for 5 days with M-CSF and compared with CM from BMMs treated with both M-CSF and RANKL for 0–3 days or 3–5 days (Fig. 8A). CM from WT, but not *Cox-2* KO, BMMs treated with both M-CSF and RANKL inhibited the PTH stimulation of *Osteocalcin* in POBs. CM from WT BMMs treated only with M-CSF did not significantly inhibit. Inhibition by CM from WT BMMs cultured for 0–3 days was similar to that from BMMs cultured for 3–5 days. The 3 day BMM culture, treated with both M-CSF and RANKL, was used in all further experiments. Some TRAP + multinucleated cells were present in both WT and KO BMM cultures treated for 3 days with M-CSF and RANKL (data not shown).

Although CM from WT BMMs inhibited PTH-stimulated *Osteocalcin* expression, WT CM did not inhibit *Osteocalcin* in vehicle-treated cultures compared to cultures without CM (Fig. 8B). In addition, CM from *Cox-2* KO BMMs had no effect on vehicle-treated POBs.

To look at the effects of CM on responses to exogenous PGE_2, we examined effects of WT and *Cox-2* KO CM on PGE_2-and PTH + PGE_2-stimulated *Osteocalcin* expression (Fig. 8C). WT CM did not inhibit PGE_2 stimulated *Osteocalcin* expression but did inhibit the stimulation of expression by PTH and PTH + PGE_2. In the presence of *Cox-2* KO CM, the combination of PTH and PGE_2 had additive effects on *Osteocalcin* mRNA, confirming that a factor (or factors) made by BMMs expressing COX-2, not only inhibited PTH-stimulated *Osteocalcin* but also caused the inhibitory interaction of PTH and PGE_2.

To confirm the role of EP4 in the inhibitory effect, we treated *Cox-2* KO POBs with CM from WT, *Ptger2* and *Ptger4* KO BMMs (Fig. 8D). PTH inhibited *Alp* expression relative to vehicle in the presence of CM from WT BMMs or *Ptger2* KO BMMs. In contrast, in the presence of CM from *Ptger4* KO BMMs, PTH stimulated *Alp* expression. Hence, it seems likely that PGs produced by BMMs acted on BMMs *via* EP4 to produce one or more soluble factors that inhibited the osteogenic effects of PTH on POBs. This observation supports the likelihood that the PG involved in the inhibitory effects is PGE_2.

Discussion

This study confirms and expands upon our previous observation that COX-2 produced PGs inhibit PTH-stimulated OB differentiation in BMSCs [26]. When COX-2 expression or PG production was absent, PTH markedly stimulated OB differentiation in BMSCs. The window for the stimulatory effect was the first week of culture, and this observation, in conjunction with similar effects of PTH on both OB and adipocyte differentiation, suggests that PTH was acting on OB precursors or MSCs, consistent with reported effects of PTH on OB precursors or MSCs *in vivo* [2,7].

Because PTH is stable in culture up to 72 h between medium changes [35], our culture conditions provided continuous exposure of

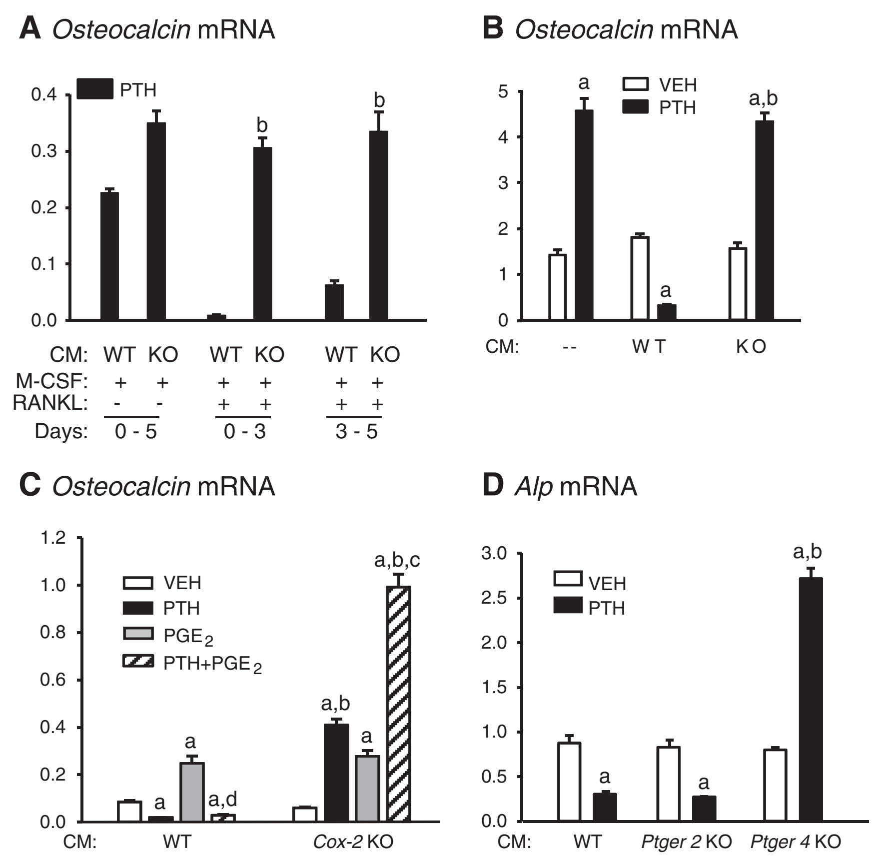

Fig. 8. Effect of conditioned media (CM) from BMM cultures on PTH-stimulated POB differentiation. POBs in all experiments were from *Cox-2* KO mice. CM were taken from BMMs treated with M-CSF (30 ng/ml) alone or with both M-CSF and RANKL (30 ng/ml). POBs ± CM were treated with vehicle (VEH), PTH (10 nM) or the combination for 14 days. All POB cultures were done in the presence of OPG (50 ng/ml) to block osteoclast formation. Gene expression was measured by qPCR. (A) Comparison of effects of CM, pooled from different periods of BMM culture, on PTH-stimulated *Osteocalcin* expression in POBs. BMMs were cultured with M-CSF only for 5 days or with both M-CSF and RANKL for 0–3 or 3–5 days. After this experiment, all further experiments used CM from BMMs cultured for 3 days with both M-CSF and RANKL. (B) Comparison of effects of WT and *Cox-2* KO CM on vehicle- and PTH-stimulated *Osteocalcin* expression in POBs. (C) Effects of WT and *Cox-2* KO CM on the ability of exogenous PGE_2 (0.1 μM) and PTH + PGE_2 to stimulate *Osteocalcin* expression in POBs. (D) Effects of CM from WT, *Ptger2* KO, and *Ptger4* KO BMMs on *Alp* mRNA expression in POBs. Bars are means ± SEM for $n = 3$. [a]Significant effect of treatment (compared to vehicle), $p < 0.01$. [b]Significant effect of genotype (compared to WT) of BMMs from which CM was obtained, $p < 0.01$. [c]Significantly different from PGE_2 and PTH alone, $p < 0.01$. [d]Significantly different from PGE_2 alone, $p < 0.01$.

cells to PTH, which in most *in vitro* studies has resulted in inhibition of OB differentiation. Because intermittent PTH is anabolic *in vivo* but continuous PTH is catabolic, it is often assumed that PTH must be applied intermittently *in vitro* in order to be osteogenic. This assumption was strengthened by positive effects on OB differentiation when cells had short, transient exposure to PTH [8,10,45]. However, the brief duration of PTH exposure is usually accomplished by removing PTH-containing media and replacing with fresh media. Since this procedure also removes PGs that accumulate in the media, it is possible that the osteogenic effects in such experiments were really due to the removal of PGs that inhibited osteogenic effects of PTH.

The inhibitory effects of PGs on OB formation did not occur in vehicle-treated BMSC cultures but only in PTH-treated BMSCs. In these cultures, OCLs were formed in response to PTH during the same "window" of time that PTH had its stimulatory effect. The inhibitory effects of PGs did not occur in POBs washed free of hematopoietic cells or in OPG-treated BMSCs. Co-cultures of POBs with BMMs or with CM from BMMs demonstrated that RANKL-treated BMMs were required to see the inhibitory effects of PGs. The need for RANKL in order to see the inhibitory effects and the reversal by OPG suggest that the BMMs involved were committed to the OC lineage. Finally, using these same co-cultures, we showed that PGs acted on BMMs to cause them to produce a soluble factor or factors that then acted on OBs to suppress PTH-stimulated OB differentiation.

We could find no precedent for a soluble factor produced in OC lineage cells in response to PGs that inhibited PTH-stimulated OB differentiation. A number of studies have shown that soluble factors produced by monocytes and non-resorbing OCs can regulate OB differentiation in a stimulatory, but not inhibitory, manner [46–51]. Osteal macrophages (osteomacs), resident macrophages in bone-lining tissues that interact with OBs, would seem to be ruled out as candidates for producing the inhibitory factor observed in our study because studies indicate that they do not become OCs (or at least do not have their regulatory functions if they commit to become OCs) [52–54]. Several studies have proposed OC-produced factors that, unlike our findings, are not specific for PTH-treated cultures but can inhibit OB differentiation in general. These factors include cardiotropin-1 [55], semaphorin 4D [56], and sclerostin [47]. We have done several microarray studies on the BMMs under our culture conditions and did not find differential expression of any of these factors by COX-2 expression/activity or PGE_2 addition (data not shown), but this does not rule out their regulation at the protein level.

The inhibition of PTH-stimulated differentiation mediated by endogenous PGs could be generated by addition of PGE_2, but not other agonists for other PG receptors, to cultures. Moreover, production of the inhibitory CM required expression on BMMs of EP4, one of two receptors for PGE_2 that activates cAMP signaling. Hence, it seems likely that the endogenous PG mediating the inhibitory action under our conditions is PGE_2. PGE_2 is expected to have its major actions *via* cAMP/PKA signaling pathways similar to those stimulated by PTH. Exogenous PGE_2 concentrations as low as 0.1 nM were sufficient to inhibit osteogenic effects of PTH, and levels $\geq$4 nM were seen in vehicle-treated co-cultures of POBs and BMMs as long as one cell type expressed COX-2. PGE_2 itself stimulates OB differentiation *in vitro*, as shown in the current studies. For a number of agents, such as TGFβ, BMP2, strontium ranelate and fresh serum [14,17–19], the induction of COX-2 expression and PGE_2 production enhances their stimulation of OB differentiation *in vitro*. In contrast to PTH, these agents all have major actions *via* signaling pathways other than cAMP/PKA. Hence, other agonists that act *via* cAMP signaling pathways might also be inhibited by PGE_2 in this culture model.

CM from COX-2 expressing BMMs did not block the stimulatory effects of endogenous PGs or exogenous PGE_2 unless the cultures were also treated with PTH. In the absence of BMMs, the combination of PTH with PGE_2 had additive effects on OB differentiation, as expected of two osteogenic agents. In contrast, in the presence of the as yet unidentified factor or factors secreted by BMMs, the stimulatory effect of the combination of PTH and PGE_2 was abrogated. Assuming that the stimulatory effects of PTH and PGE_2 on OBs are mediated *via* stimulation of cAMP, it is possible that the CM contains a factor that acts *via* $G\alpha_i$ to inhibit production of PTH- and PGE_2-stimulated cAMP. PGE_2 in WT CM can act *via* EP3, which is coupled to $G\alpha_i$. However, it is unclear why this effect would only occur in the presence of PTH. The factor that blocks PTH-stimulated differentiation produced by BMMs is unlikely to be PGE_2 itself because the addition of PGE_2 to PTH, in the absence of BMMs or WT CM, resulted in additive stimulatory effects. Another possibility is that PTH induced activity of a phosphodiesterase that rapidly degraded PGE_2-stimulated cAMP, but it is unclear why this should happen only in the presence of WT BMMs or CM. Although the explanation awaits further studies, this observation might explain why it has also been difficult to demonstrate an anabolic effect of systemically applied PGE_2 in mice [57]. Because the inhibitory factor made by BMMs blocks the stimulatory effects of PGE_2 in the presence of PTH and because endogenous PTH is present continuously *in vivo*, PGE_2 given *in vivo* might act on BMMs to suppress not only PTH-stimulated OB differentiation but also its own ability to stimulate OB differentiation.

In our *in vitro* study, PGE_2 is stable in the media (personal observation), unlike the conditions expected *in vivo*. PGs *in vivo* are not stored but are synthesized, released as needed and rapidly metabolized in their passage through the lung [58]. COX-2 protein is estimated to have a half-life on the order of 2 h [59,60], and the local level of PGs *in vivo* is highly dependent on new production of *Cox-2*, which is a rapidly inducible and transiently expressed gene [14]. However, even when PTH was given intermittently, where the interaction of PTH and PGE_2 is expected to be brief, we found that PTH *in vivo* was more anabolic in *Cox-2* KO mice than in WT mice [25]. A more marked effect of the inhibitory interaction of PTH and PGs on OB differentiation is expected in the continuous PTH infusion protocol, because both PTH and PGs should be continuously elevated. In addition, there should be an abundance of OCs generated by continuous PTH *in vivo* to produce the inhibitory factor(s). It is possible, therefore, that the PTH induction of COX-2 could account for some of the bone loss seen with continuous PTH *in vivo*.

Our findings suggest a novel role for COX-2 produced PGE_2 *in vitro* to inhibit PTH-stimulated osteogenic/anabolic activity *via* actions through EP4 on early osteoclastic lineage cells. PGE_2 is likely to be generated by COX-2 induction in many types of culture, and these findings suggest that it may have important modulatory roles that are overlooked. A better understanding of how PGs modulate the actions of PTH may help us be more effective in targeting bone remodeling for the treatment of osteoporosis and lead to the future development of new anabolic agents or protocols to improve therapy for osteoporosis and other skeletal defects.

Acknowledgments

We owe much to Larry Raisz who never wavered in his belief that prostaglandins were important for bone biology. We are also grateful to the reviewers of this manuscript for helping us to clarify our thoughts about the PTH–PGE_2 interaction. This work was supported by NIH grants R56DK048361, AR047673 and AR060286.

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